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Phosphorylation of serine residues in endogenous proteins of thylakoids and subthylakoid particles in the dark under nonreducing conditions

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Isolated thylakoids, Photosystem I and Photosystem II particles were phosphorylated with [γ-^{32}P]ATP at high specific radioactivity in the dark under nonreducing conditions and in the light in the absence of electron acceptor. The resulting phosphoproteins were compared by gel electrophoresis and autoradiography. Phosphorylation of thylakoids in the dark and in the light rendered distinct patterns of phosphoproteins. Some of the dark-phosphorylated proteins in thylakoids were diminished or not detected in the light-phosphorylated membranes. Phosphorylation of subthylakoid particles was insensitive to light and most of the phosphoproteins in these membranes were also observed in the dark-phosphorylated thylakoids. Dark phosphorylation rendered mostly phosphoserine in individual proteins of thylakoids, subthylakoid particles and lysine-rich histone phosphorylated by the particles. Conversely, phosphothreonine was prevalent in light-phosphorylated thylakoids. The results are consistent with the presence of a protein serine kinase activity that is distributed homogeneously within the thylakoid regions, is more active in the dark, does not require reducing conditions for activity and phosphorylates a number of endogenous substrates most of which belong to the stroma membranes.

Introduction

Phosphorylation of the light-harvesting, chlorophyll *a/b* protein of the photosystem II complex (LHC II) in higher plant chloroplasts [1] is part of the state transition mechanism triggered when PS II reaction center is overexcited with respect to PS I [2]. Furthermore, PS II components such as CP 43 apoprotein, D_1, D_2 and a 10–11 kDa protein were unambiguously demonstrated to be phosphorylated under those conditions [3,4]. The phosphorylation of some of these PS II components may be involved in the photoinactivation observed at high light intensity [5]. Some evidence suggests that the phosphorylation of all these proteins is catalyzed by the same light-activated, threonine protein kinase [6,7]. The enzymology of these phosphorylations was revealed when Coughland and Hind reconstituted LHC II phosphorylation in situ and in vitro with a 64 kDa protein kinase purified from thylakoids [6,7]. LHC II kinase undergoes autophosphorylation and is localized together with LHC II and PS II core complex in the grana region of thylakoids [8].

Before these findings were made, two protein kinases specific for serine residues: $ChlPK_1$ of 25 kDa and $ChlPK_2$ of 38 kDa had been isolated from thylakoids [9,10]. Neither of these kinases phosphorylated LHC II proteins in vitro [10].

While most of the research performed in this field contributed greatly to our understanding of the light-activated phosphorylation of thylakoid proteins, little attention was focused on the dark phosphorylation. In *Chlamydomonas reinhardtii* it was clearly demonstrated that some of the PS I components are phosphorylated in the dark [11]. Under similar experimental conditions no phosphorylation of PS I components was detected in spinach, wheat and pea thylakoids [11]. Recently we reported [12] that a dark protein serine kinase and a light protein threonine kinase can be functionally dis-

Abbreviations: P_i, inorganic phosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; $ChlPK_1$, chloroplast protein kinase 1; $ChlPK_2$, chloroplast protein kinase 2; CP, chlorophyll protein complex of Photosystem I and Photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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tinguished in isolated thylakoids by using lysine-rich histone as exogenous substrate.

Here we show that when [γ-^{32}P]ATP at high specific radioactivity (> 1000 cpm/pmol) is used in the kinase assay, a dark, redox-independent protein serine kinase activity can be detected in thylakoids and in subthylakoid particles. This kinase activity seems to be homogeneously distributed in the thylakoid membrane, where it phosphorylates a number of distinct endogenous proteins in the dark, mostly of the non-appressed stroma region.

Materials and Methods

Materials

Phosphoserine, phosphothreonine, lysine-rich histone III and Triton X-100 were from Sigma (U.S.A.). Digitonin from Sigma was recrystallized twice in ethanol. Carrier-free [^{32}P]P_i was from Dupont. Salt-free, carrier-free [γ-^{32}P]ATP was prepared as described in Ref. 13. Spinach (*Spinacia oleracea* L.) plants were grown under environmentally controlled conditions as described in Ref. 12.

Isolation of thylakoid membranes, PS I and PS II particles

Thylakoid membranes were prepared from spinach leaves that were kept in the dark at room temperature for 30 min [12] and purified by discontinuous 0.9–1.8 M sucrose gradient [14] as described. PS I [15] and PS II [16] particles were prepared from sucrose gradient purified thylakoids as described. Chlorophyll concentration was determined as in Ref. 17. For some experiments thylakoids from intact chloroplasts or from percoll gradient-purified intact chloroplasts [18], were obtained as described [19].

Phosphorylation of the isolated membranes and protein kinase assay

For the phosphorylation of the endogenous proteins freshly prepared thylakoids or PS I or PS II particles were incubated in a medium (50 μl) containing 5 mM $MgCl_2$, 75 mM Tris-HCl (pH 7.5), 50 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol) and membranes corresponding to 50 μg of chlorophyll for 15 min in the dark under nonreducing conditions or in the light ($7 \cdot 10^{15}$ quanta $\cdot$ $m^{-2} \cdot s^{-1}$) at 30 °C. The reaction was terminated by addition of 5 μl 0.4 M ATP and aliquots (25 μl) were either dispersed in equal volumes of sample buffer for SDS-PAGE analysis or spotted on Whatman 3MM paper (2 × 2 cm) to assay protein kinase as described [19]. Protein kinase activity on lysine-rich histone (10 μg) was measured under the experimental conditions described [12]. Protein was determined as in Ref. 9.

SDS-PAGE, autoradiography and amino acid analysis

Phosphorylated thylakoids, PS I and PS II particles were resolved by 15% SDS-PAGE [9] and the endogenous phosphoproteins, detected by autoradiography [9], were recovered by elution of the gel slices with $(NH_4)_2CO_3$ [20]. Phosphoproteins were hydrolyzed and their phosphoamino acids were resolved by high-voltage paper electrophoresis and quantitated as described [9].

Results

Under nonreducing conditions in the dark a protein kinase activity on endogenous substrates with a K_m for ATP of 20 μM and a specific activity of 14 pmol ATP/mg protein per min is detected in isolated thylakoids. Illumination with actinic light in the absence of electron acceptor activates a protein kinase that phosphorylates endogenous substrates with a K_m for ATP of 80 μM and a specific activity of 48 pmol ATP/mg protein per min, in accordance with a previous report on the phosphorylation of lysine-rich histone as exogenous substrate [6].

A striking difference between the phosphorylation of isolated thylakoids in the light and in the dark was observed when the corresponding patterns of phosphoproteins were compared (Fig. 1, lanes 1 and 4). It is worth mentioning that the dark phosphorylation pattern shown in Fig. 1 could be detected only when

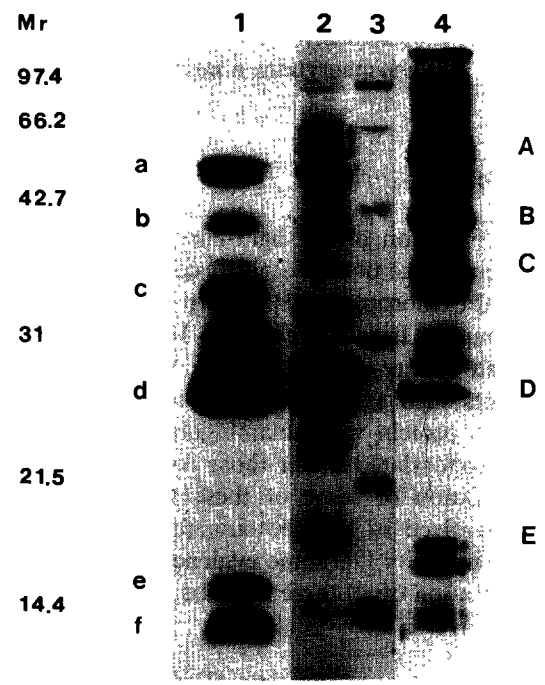

Fig. 1. Autoradiography of the SDS-PAGE resolved thylakoids after phosphorylation of the thylakoids with [γ-^{32}P]ATP in the light or in the dark. Lane 1, autoradiography of the light-phosphorylated membranes; lane 2, Coomassie-blue-stained thylakoid proteins and, lane 3, of molecular mass standards corresponding to 97.4, 66, 42.7, 31, 21.5 and 14.3 kDa from top to bottom; lane 4, autoradiography of the dark-phosphorylated membranes. Letters by lanes 1 and 4 identify those phosphoproteins, the phosphoamino acid composition of which have been determined and are presented in Table I.

phosphorylation was carried out with [γ-^{32}P]ATP at a specific radioactivity of 1000 cpm/pmol or higher. While the light phosphorylation pattern was still detected with [γ-^{32}P]ATP at a specific radioactivity of 200 cpm/pmol, the dark phosphorylation pattern was negligible (data not shown). This may explain why the dark phosphorylation pattern was not detected before, since the specific radioactivity of [γ-^{32}P]ATP currently used in studies on thylakoid protein phosphorylation is in the range of 100–300 cpm/pmol. It is well documented that when thylakoids are phosphorylated in the light in the absence of electron acceptor, the major substrates are LHC II (25 kDa) and other proteins associated with the PS II complex [1,3]. Thus, phosphoproteins of 58 (a), 42 (b), 32 (c), 25 (d), 16 (e) and 10 (f) kDa are detected in light-phosphorylated thylakoids (Fig. 1, lane 1). Phosphorylation of isolated thylakoids in the dark under nonreducing conditions (Fig. 1, lane 4) rendered phosphoproteins of 63 (A), 42 (B), 38 (C), 26 (D) and 18 (E) kDa, some of which (A and E) were not even detected when the phosphorylation was carried out in the light (Fig. 1, lane 1).

Individual phosphoproteins from light-phosphorylated (Table I, lower-case letters) and from dark-phosphorylated thylakoids (Table I, capital letters) described in Fig. 1, were eluted from the gel and their phosphoamino acid composition was determined. In agreement with previous reports, the majority of the light-phosphorylated proteins analyzed in Fig. 1 carry their phosphate group preferentially in threonine residues. However, a low level of phosphoserine was also detected in all of them (Table I). The ratio phosphoserine/phosphothreonine varied from 0.1 to 1.2 in individual thylakoid proteins phosphorylated in the light. Conversely, in dark-phosphorylated proteins the phosphoserine/phosphothreonine ratio varied from 3.7 to 10.6, suggesting that phosphoserine is indeed prevalent in dark phosphorylation under nonreducing conditions.

TABLE I

Phosphoamino acid analysis of thylakoid proteins phosphorylated in the light and in the dark

Phosphoamino acid analysis of individual phosphoproteins from light-phosphorylated (lower case letters) and from dark-phosphorylated (capital letters) thylakoids as shown in Fig. 1. In parentheses is the mass of each phosphoprotein (kDa). Numbers indicate cpm recovered in each phosphoamino acid spot upon resolution by high-voltage electrophoresis.

Phosphoprotein	P-Ser	P-Threo	P-Ser/P-Threo
Light-phosphorylated thylakoids			
a (58)	188	470	0.4
b (42)	140	114	1.2
c (32)	168	598	0.3
d (25)	316	2350	0.1
e (16)	294	1195	0.2
Dark-phosphorylated thylakoids			
A (63)	518	49	10.6
B (42)	648	175	3.7
C (38)	548	67	8.2
D (26)	594	96	6.2
E (18)	380	42	9.0

TABLE II

Dark serine protein kinase activity in endogenous substrates of thylakoids obtained by different procedures

Thylakoids were prepared and dark protein kinase on endogenous substrates was assayed as described under Materials and Methods.

Thylakoids obtained from:	Kinase activity (pmol ATP/mg protein per min)
Broken chloroplasts without purification	4.4
Broken chloroplasts and purified by sucrose gradient	11.2
Intact chloroplasts	12.0
Percoll gradient-purified intact chloroplasts	11.6

It is interesting to note that the 42 kDa (b) protein showed a higher level of phosphorylation in serine than in threonine residues both in the dark and in the light and that illumination inhibits serine phosphorylation (Table I). These results agree with previous observations that thylakoids phosphorylate lysine-rich histone in serine and in threonine residues in the light but only in serine residues in the dark and that serine phosphorylation is impaired by light [12].

Since thylakoids are prepared from broken chloroplasts the possibility exists that the dark protein kinase activity could be a contaminant from other chloroplasts or subcellular compartment. To study this possibility we measured dark protein kinase activity in thylakoids that were prepared following different purification protocols. Sucrose gradient-purified thylakoids from broken chloroplasts, thylakoids from intact chloroplasts and thylakoids from Percoll gradient purified intact chloroplasts showed a phosphoprotein pattern similar to that described in Fig. 1, lane 4, and the highest protein kinase activity in the dark under nonreducing conditions (Table II). Thylakoids from broken chloroplasts without further purification showed the lowest level of protein kinase activity (Table II).

Some thylakoid proteins are heterogeneously distributed within the thylakoid membrane [21]. Although there is a lateral mobility [22], PS I components and the coupling factor 1 are localized at the non-appressed stroma membrane while PS II components are localized at the appressed grana membranes [23]. Conversely, cytochrome b_6/f is homogeneously distributed [24]. By treatment of the thylakoids with digitonin [15] or with Triton X-100 [16] and subsequent purification, it is possible to obtain particles enriched in PS I and PS II components, respectively. These particles have been successfully used to determine the lateral distribution of

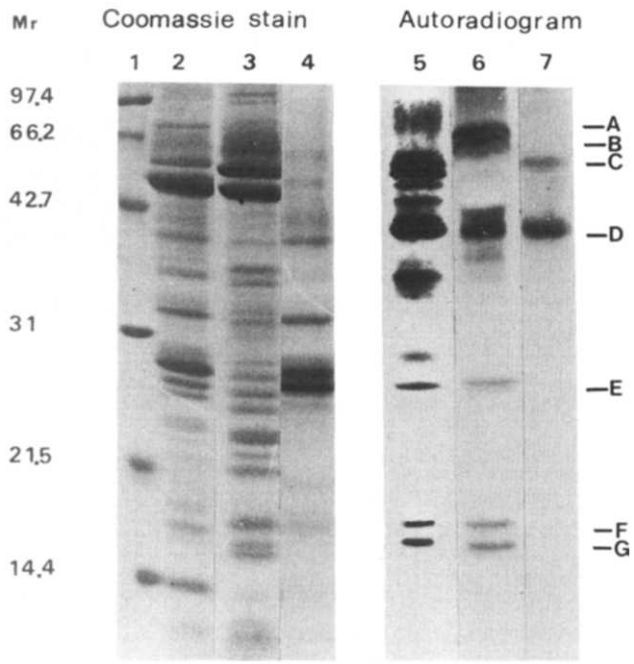

Fig. 2. Autoradiography of the SDS-PAGE-resolved proteins of PS I and PS II particles after phosphorylation with [γ-^{32}P]ATP in the dark. Lanes: 1, molecular weight standards as in the legend to Fig. 1; 2, thylakoids; 3, PS I particles; 4, PS II particles; 5, thylakoids; 6, PS I particles and 7, PS II particles. Letters to the right indicate the position of the phosphoproteins detected in the particles.

LHC II kinase which is located only at the appressed regions of thylakoids associated to PS II and LHC II components [8]. When isolated PS I and PS II particles were phosphorylated in the dark, the pattern of serine phosphoproteins shown in Fig. 2 was observed. Six phosphoproteins, of 72 (A), 70 (B), 42 (D), 26 (E), 18 (F) and 16 (G) kDa were detected in PS I particles. The pattern was simpler in PS II particles, where only two phosphoproteins, of 63 (C) and 42 (D) kDa, were detected. Bands D, E, F and G of PS I particles and bands C and D of PS II particles are also present in the dark-phosphorylated thylakoids.

Using lysine-rich histone as exogenous substrate we determined that the dark, protein serine kinase activity is 2.7-fold enriched in PS I particles (Table III). This result also shows that the dark serine kinase activity is

TABLE III

Distribution of the redox independent dark serine protein kinase activity in thylakoid and subthylakoid membranes

Lysine-rich histone was phosphorylated by thylakoids and by subthylakoid particles in the dark as described under Materials and Methods.

Membrane	Kinase activity (pmol ATP/mg protein per min)
Thylakoids	9.5
Photosystem I particles	26.0
Photosystem II particles	1.9

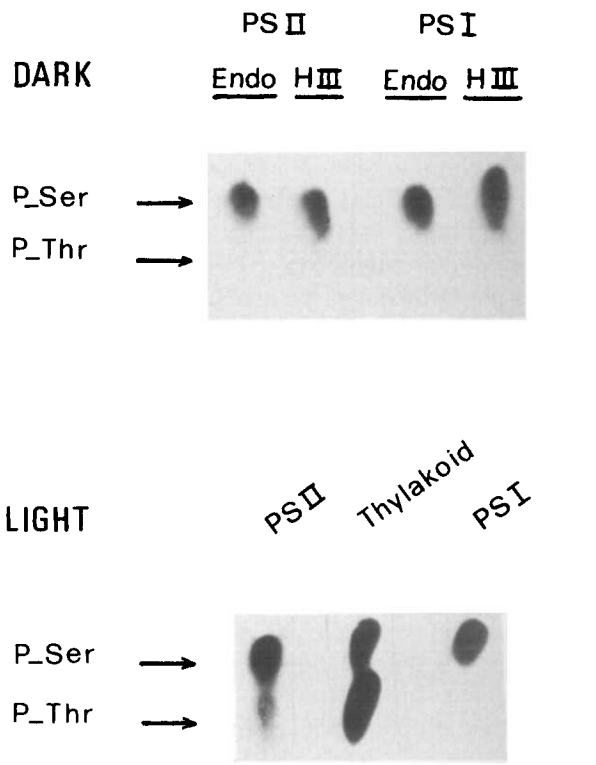

Fig. 3. Phosphoamino acid analysis of: endogenous proteins (Endo) in PS I and PS II particles and in histone III (H III) phosphorylated in the dark, and of endogenous proteins in thylakoids, PS I and PS II particles phosphorylated in the light.

not inactivated by detergent fractionation of the thylakoids.

Phosphoamino acid analysis revealed only phosphoserine in endogenous substrates of the particles and in lysine-rich histone phosphorylated by the particles in the dark (Fig. 3). The pattern of phosphoproteins in PS I and in PS II particles was not altered by illumination (not shown). However, when lysine-rich histone was phosphorylated by the particles in the light, only PS II particles catalyzed a low level of threonine phosphorylation: under identical conditions, thylakoids catalyzed the phosphorylation of threonine residues in lysine-rich histone 10-times faster (3900 cpm/15 min) than PS II particles (400 cpm/15 min) (Fig. 3). Thus, phosphoamino acid analysis confirms previous observations that the activation of the redox controlled threonine kinase is sensitive to detergent fractionation [25] and that the kinase is localized in the grana thylakoid [8].

Discussion

Phosphorylation of isolated thylakoids with [γ-^{32}P]ATP at high specific radioactivity (> 1000 cpm/pmol) showed a distinct pattern of dark phosphoproteins (Fig. 1). The 15 min incubation time used in the kinase assay for the results shown in Fig. 1 may

overestimate dark phosphorylation with respect to light phosphorylation, since the light-activated protein kinase reaction is not linear for more than 5 min. However, the assay permitted detection of those thylakoid proteins that are phosphorylated under conditions where LHC II kinase is not active, that is, in the dark under nonreducing conditions. The possibility that this dark protein serine kinase is a contaminant from other cellular compartments is unlikely, since thylakoids from broken chloroplasts that were not further purified showed a lower kinase activity than sucrose-gradient-purified thylakoids from Percoll-gradient-purified intact chloroplasts (Table II). Dark phosphorylation of PS I and PS II particles at high specific radioactivity of [γ-^{32}P]ATP also produced a distinct pattern of phosphoproteins (Fig. 2). Dark phosphorylation took place in serine residues of endogenous thylakoid and subthylakoid particles and of lysine-rich histone phosphorylated by the particles (Fig. 3).

At least four dark-phosphorylated proteins of PS I particles and the two dark-phosphorylated proteins of PS II particles are also observed in thylakoids. The fact that the majority of the dark phosphoproteins in subthylakoid particles are also detected in sucrose gradient-purified thylakoid is another argument against a contaminant protein kinase. The presence of the 63 kDa phosphoprotein in thylakoids and in PS II particles suggests that this substrate and the corresponding kinase are localized in the grana region. The 63 kDa phosphoprotein was also observed when samples were not heated before SDS-PAGE analysis, suggesting that it is not an aggregated form of a lower-molecular-weight species. The presence of a 63 kDa protein in PS II particles is intriguing, since no protein with that mobility was reported in highly purified particles [26]. Therefore, the 63 kDa phosphoprotein could be a contaminant in PS II particles prepared according to Berthold et al. [16]. However, it is suggestive that the 63 kDa phosphoprotein does not overlap a sharply stained band (Fig. 2). In thylakoid membranes some proteins fail to bind Coomassie blue and others are even insensitive to silver staining [27]. Moreover, some proteins can be readily detectable only by phosphorylation [28]. To confirm that this is the case for the 63 kDa phosphoprotein in PS II particles, further experiments using highly purified particles and two-dimensional gel electrophoresis analysis as described by Ikeuchi et al. [26] are required. In the case of the 42 kDa phosphoprotein that is detected in thylakoids, in PS I and in PS II particles it appears that the substrate and the kinase are homogeneously distributed within the thylakoid disk. Detergent fractionation of thylakoids seems to be suitable to preserve dark serine kinase activity, since a 2.7-fold enrichment of this activity is observed in PS I particles (Table III). These results can be best interpreted assuming the existence of a serine protein kinase activity, perhaps composed of more than one kinase, that is distributed homogeneously within the thylakoid disk but mostly enriched in the stroma lamellae (Table III), where it phosphorylates a number of phosphoproteins, in the dark (Fig. 2).

Upon light activation, the membrane-bound LHC II kinase recognizes preferentially threonine residues of endogenous thylakoid substrates [1] and of lysine-rich histone as exogenous substrate [12,29]. However, the background of phosphoserine detected in all the light-phosphorylated proteins (Fig. 1) may also be due to LHC II kinase activity, since the purified enzyme was shown to phosphorylate serine residues in lysine-rich histone [26]. Hence, it is reasonable to expect that LHC II kinase can randomly phosphorylate serine residues of endogenous substrates at a low rate. Light-dependent serine phosphorylation in some thylakoid proteins is less than 10% of the threonine phosphorylation (Fig. 1), suggesting that such an event could indeed be a side-reaction of LHC II kinase. It is not clear why the ratio phosphoserine/phosphothreonine is not constant for all the light-phosphorylated thylakoid proteins. An interesting possibility is that upon light activation the membrane-bound LHC II kinase activates other kinase(s) that in turn phosphorylate substrates only in the light. If that were the case, the inactivation of LHC II kinase would prevent light phosphorylation in all thylakoid proteins as it was shown by treatment of the thylakoids with antibodies against LHC II kinase [7].

The mechanism by which illumination impairs serine phosphorylation of endogenous thylakoid proteins (Fig. 1) and of lysine-rich histone as exogenous substrate [12] is unknown. A trivial explanation is that the activation of LHC II kinase promotes a competition by ATP with the dark serine protein kinase. However, this possibility is unlikely, since light-inhibition of serine protein phosphorylation is also observed in fresh thylakoids in the presence of DCMU and in frozen-thawed thylakoids that lack the light-activated protein kinase [12]. Moreover, under our experimental conditions the light-activated protein kinase drops the concentration of ATP from 50 μM to 45 μM. At both concentrations of ATP the specific activity of dark protein kinase was the same (data not shown). Light inactivation is observed only in thylakoid, and not in subthylakoid, particles, suggesting that the integrity of the thylakoid is required. It is worth recalling that the light activation of LHC II kinase is sensitive to detergent fractionation of the membranes [25] (Fig. 3). Therefore, this could also be the case for the light inactivation mechanism of protein serine phosphorylation. Further studies are required to determine whether the light inactivation on serine protein kinase is coupled to the redox state of some thylakoid electron carrier.

Light phosphorylation of isolated PS II particles that were obtained by mechanical fractionation rendered a

phosphoprotein pattern identical to that observed in thylakoids [25]. Moreover, Coughland and Hind have presented strong immunological evidence showing that LHC II kinase is located only in the appressed grana membrane with PS II proteins [7]. Therefore the low level of phosphothreonine detected in lysine-rich histone phosphorylated by PS II particles in the light (Fig. 3) is due to a residual light activation of LHC II kinase upon fractionation of the membranes with detergent.

An important aspect of the results presented here is that isolated PS I particles representing the non-appressed stroma membrane possess protein kinase(s) preferentially specific for serine residues that phosphorylate at least six endogenous proteins and lysine-rich histone in the dark.

The concept that several kinases may be involved in thylakoid protein phosphorylation is reinforced by the fact that two kinases have been purified from thylakoids, one of which, $ChlPK_1$ of 25 kDa, was confirmed [30]. We are now raising monospecific antibodies against $ChlPK_1$ to gain insight into the location of this kinase within the thylakoid regions. Appropriate experiments of reconstitution, similar to those reported by Coughland and Hind [7] with purified LHC II kinase, will allow us to determine whether purified $ChlPK_1$ is involved in the phosphorylation of some of the dark-phosphorylated proteins of thylakoids. Identification of the dark-phosphorylated 42 kDa protein may provide some clues as to the possible role of the serine protein kinase reported here. The interesting possibility of cross-talk among kinases in thylakoids can not be ruled out.

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